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Sex identification in birds using two CHD genes

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SUMMARY

In theory, birds should control the sex ratio of the offspring they produce. In practice, we have very limited evidence to support this idea because of our difficulty in sexing nestling birds. In addition, extinction is facing an increasing number of birds. Our ability to help includes captive breeding which, again, is difficult if male and female adults cannot be recognized.

Here we describe the discovery of a W-linked gene in the Great tit (*Parus major*). It is named CHD-W (chromodomain-helicase-DNA-binding protein W-linked), it is highly conserved and it is W-chromosome linked in a range of bird species. These birds also possess a second, non-W-linked CHD gene (CHD-NW). A single, simple polymerase chain reaction technique based on both genes can be used to identify the sex in a wide variety of birds.

1. INTRODUCTION

There is no rapid, general method for determining the gender of birds. In many species adults cannot be sexed by their appearance. Futhermore, only a tiny proportion of birds can be sexed as chicks. A general sexing technique would have two primary uses: the evaluation of sex ratio theories, and the management of endangered species.

Sex ratio theory predicts that the proportion of males produced should vary adaptively in animal populations with the prospects for male offspring as compared with female offspring (Trivers & Willard 1973). In recent years, several sexing techniques have been used to show that adaptive sex ratios in birds may be far from rare. Significant deviations from parity have been reported as a function of time of year (Howe 1977; Dijkstra *et al.* 1990; Zijlstra *et al.* 1992; Daan *et al.* 1996), of egg sequence (review Krackow 1995) and of territorial quality (Komdeur 1996).

Most of these studies depend on those species whose chicks can be sexed by external characteristics. This can only be done when the chicks are fairly old and nestling mortality has already occurred. This raises the question whether sex ratio bias emerges as a by-product of differential mortality (Clutton-Brock 1986; Weatherhead & Teather 1991; Dijkstra & Daan, submitted), or is already present at oviposition (Howe 1977; Dijkstra *et al.* 1990). This question has had to be addressed in other ways: by embryonic dissection (Howe 1977), by establishing sex ratio change with order of laying (Dijkstra *et al.* 1990), or by analysis of blood samples collected at hatching with a species-specific DNA technique (Griffiths & Tiwari 1993; Komdeur 1996). These methods require considerable expertise and must be modified between species. Furthermore, the sex ratio bias reported is usually restricted to a range of 40–60 % males, with few exceptions (Komdeur 1996). Statistical certainty in the detection of such deviations from 50 % requires that large numbers of offspring are analysed so it would greatly increase efficiency if sexing could be done at hatching rather than fledging. The situation calls for a rapid method for sex identification that is readily applicable to any avian species. Indeed with evidence for primary sex ratio adjustment in avian reproduction, a quest for the mechanism will arise which necessarily requires the assessment of gender in the developing egg before laying.

The second reason to develop a generally applicable sex assay is for adults of rare monomorphic species. The Spix's macaw (*Cyanopsitta spixii*) provides a case in point. From 1990 to 1995 only a single individual of unknown sex was left in the wild. The sex of the wild bird was determined from the DNA in moulted feathers and a potential female mate released (Griffiths & Tiwari 1995). Unfortunately, about 20 % of the worlds bird species are either threatened or near threatened with extinction (Collar *et al.* 1995) so a widely applicable method of DNA sex identification is urgently needed.

The DNA based sexing system described here employs a W-linked gene discovered in the Great tit. This gene is conserved and W linked in examples from across the Avian class. A single set of polymerase chain reaction (PCR) primers are presented that we demonstrate will correctly sex a wide variety of bird species.

2. METHODS

DNA was isolated from blood taken from chicken (*Gallus domesticus*), great tit, (*Parus major*) marsh harrier (*Circus aeruginosus*), kestrel (*Falco tinnunculus*; all previous species

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sexed by adult plumage) bee-eater (*Merops apiaster*; plumage behaviour), hyacinth macaw (*Anodorhynchus hyacinthinus*), spix's macaw (chromosome analysis), boobook owl (*Ninox novaesiae*), white-faced owl (*Ptilopsis leuctis*), burrowing owl (*Speotyto cumcularia*), Eurasian eagle owl (*Bubo bubo*), long-eared owl (*Asio otus*), tawny owl (*Strix aluco* adult size), lesser black-backed gull (*Larus fuscus* DNA (Griffiths & Holland 1990)), marbled murrelet (*Brachyramphus marmoratus*' gonadal examination), starling (*Sturnus vulgaris*: DNA (Griffiths *et al.* 1992)) and African marsh warbler (*Acrocephalus baeticatus*; reproductive behaviour). Blood was added to an equal volume of BLB (Bill's lethal brew: 2% sodium dodecyl sulphate (SDS), 50 mM EDTA, 50 mM Tris(pH8)) and then stored in the freezer.

Female great tit genomic DNA from three individuals was partly *MboI* digested and size selected (9–23 kb) on an agarose gel. A library was constructed in lambda FixII (Stratagene), plated, transferred to Zetaprobe GT (Bio-Rad) and hybridized to GT1, a 724 bp clone of the Great tit W-linked PCR product (Griffiths & Tiwari 1993). Hybridization occurred in 5% SDS–0.25 M Na_2HPO_4 (pH 7.2) for 16 h at 65 °C and was followed by 3 × 30 min washes at 65 °C in 0.5 × SSC (1 × SSC is 0.15 M NaCl–15 mM $Na_3C_6H_5O_7.2H_2O$ (pH 7)) and 0.1% SDS. A total of two positive clones were cut with *EcoRI* and the inserts subcloned into *EcoRI* sites of pUC18 (Pharmacia) for easy handling. The Erase-a-Base kit (Promega) was used to generate nested deletions. Sequencing used T7 DNA polymerase and 7-deaza dGTP kit (USB). Screening of chicken cDNA libraries is detailed elsewhere (R. Griffiths, unpublished data).

Southern blots were prepared from genomic DNA digested with *PvuII* (BRL), electrophoresed through a 0.75% agarose gel and blotted onto Zetaprobe GT. The probe was the entire 1.3 kb insert of a CHD-W cDNA (spans 2670–4003 nucleotides in the related mouse (*Mus musculus*) CHD1 gene (Delmas *et al.* 1993)). Hybridized as above at 60 °C then washed 2 × 30 min at 45 °C in 1 × SSC–0.1% SDS before exposure for 9 d at −70 °C with two intensifying screens.

Spix macaw DNA samples were subject to PCR and the products sequenced as recorded previously (Griffiths & Tiwari 1995). PCR amplification of genomic DNA of other bird species and human was done on a Hybaid Omnigene. Reaction volumes of 20 µl were made up of Promega *Taq* buffer (1 × is 50 mM KCl, 10 mM Tris.HCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100), 200 µM of each dNTP, P2 (5′-TCTGCATCGCTAAATCCTTT) and P3 (5′- AGATATTCCGGATCTGATA) primers (approx. 1 µM), 50–200 ng of genomic DNA and 0.15 units of *Taq* polymerase. The thermal treatment was 94 °C per 1.5 min followed by 30 cycles of 55 or 56 °C per 15 s, 72 °C per 15 s, and 94 °C per 30 s with a finish of 56 °C per 1 min and 72 °C per 5 min. *HaeIII* (5 units; Promega) was used to cut 8 µl of PCR product in 1x Promega restriction enzyme buffer 3 and 50 ng μl^{-1} bovine serum albumin (Sigma) in a total volume of 10 µl. The digests and uncut PCR product were precipitated before being electrophoresed in a visigel (Stratagene) with ethidium bromide (40 ng ml^{-1}) at 3.5 V cm^{-1}.

3. RESULTS

Birds can be sexed from DNA by showing the absence (male: ZZ) or presence (female: ZW) of the female specific W chromosome. At the molecular level this is done by the recognition of a W-linked marker. This can only be done after a W chromosome DNA marker is identified in the avian species.

A Great tit W-linked sequence was identified after screening randomly selected primers for their ability to amplify sex linked sequences. The 724 bp W-linked DNA fragment (GT1) that was produced was isolated and used to probe a Southern blot of genomic DNA from the great tit. This showed hybridization to a fragment of female DNA with an intensity that suggested the GT1 target existed in either a single or low copy number (Griffiths & Tiwari 1993). DNA fragments that have been isolated from the W-chromosome are usually repetitive (Tone *et al.* 1982; Griffiths & Holland 1990; Rabenold *et al.* 1991), so a single copy sequence prompted further investigation.

A great tit genomic lambda FixII library was constructed and hybridized to the GT1 probe. A total of two clones hybridized intensely, each end of the insert was sequenced and the inserts were removed with *EcoRI* and subcloned in pUC18. The DNA sequence of the GT1 probe was already known to possess an *EcoRI* site so sequence determination rapidly showed the 1.8 and 8 kb subclones of clone 1 were divided at this cutting site. The 1.8 kb subclone and 3.5 kb of the 8 kb subclone were sequenced in a single direction and amino acid translations were used to screen the GenBank and EMBL data banks in April 1993. A 41 amino acid region showed a remarkable 85% identity to the CHD1 gene isolated from the mouse (chromatin organization modifier (chromo)-helicase–ATPase-DNA binding protein-1; 1286–1326 amino acids, see Delmas *et al.* 1993).

The 8 kb subclone was treated with restriction enzymes *SacI* and *HindIII* to produce a fragment of 441 bp which included the portion coding for the 41 amino acid region. This was subcloned (GT2) and used to probe chicken cDNA libraries producing clones of two closely related genes. The sequence similarity of these to the CHD1 gene (Delmas *et al.* 1993) suggested that both are homologous avian CHD genes (R. Griffiths, unpublished data). A genomic Southern blot featuring the chicken, bee-eater and hyacinth macaw was hybridized at relatively high stringency to a 1.3 kb insert of a cDNA clone from one of the chicken genes.

The autoradiograph in figure 1 shows that a similar result is apparent in each of the three species. There are two classes of bands. The first class occurs only in the female birds where 2–5 bands appear in each species. This demonstrates at least one W-linked CHD gene occurs in each. A second class of 2–4 bands occur at a similar size in both sexes of each species demonstrating the presence of a second CHD gene that is not W linked. The low number of fragments that are visible in the autoradiograph suggest that only a few genes have been located, possibly just two. The latter result would be consistent with the number of CHD genes isolated from the chicken cDNA library. We shall refer to the two avian genes in each bird as CHD-W being the W-linked gene while CHD-NW is the non-W-linked gene.

The class Aves is generally split into two major infraclasses reflecting a pair of monophyletic clades (Sibley *et al.* 1988). The largest is the Neoaves represented here by the bee-eater and the Hyacinth macaw while the second is the Eoaves represented by the chicken. That members of both infraclasses contain the same W-linked gene suggests that this gene was in place upon this chromosome before the diversification

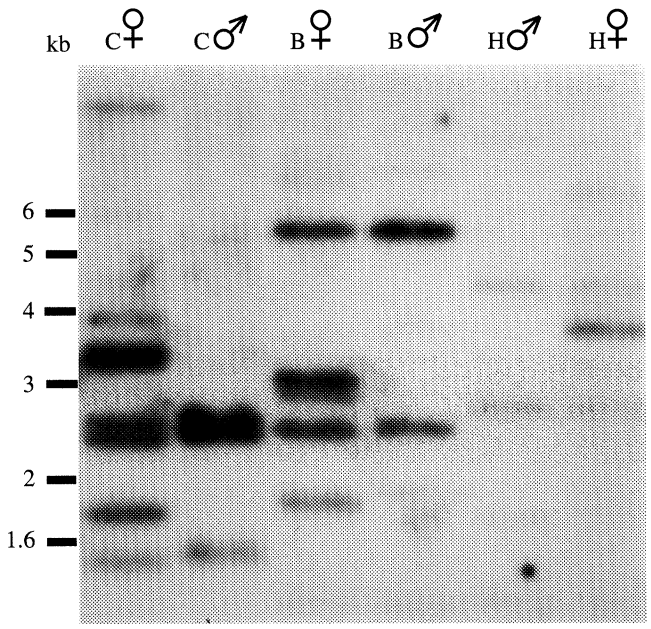

Figure 1. A Southern blot analysis of *PvuII* cut genomic DNA from male (M) and female (F) chicken (C), bee eater (B) and Hyacinth macaw (H) probed with a 1.3 kb chicken CHD-W cDNA clone. It shows that two classes of related sequences exist in all species, one is W linked while the second occurs in both sexes so is either Z or autosomally linked. Numbers to the left indicate size in kilobase (kb) pairs.

of this class. This suggests that CHD-W is likely to be W linked in all birds.

The hybridization to CHD-W and CHD-NW in the Hyacinth macaw suggested that these genes could be used to sex the closely related Spix's macaw. The only available DNA from the last wild Spix's macaw came from moulted feathers. This meant the quantity and quality of the DNA would be low so a PCR based technique would have to be used. The PCR primers were designed to amplify both CHD-W and CHD-NW through comparison of homologous fragments of cDNA sequences from the mouse (Delmas *et al.* 1993), the two chicken genes and a genomic clone of Hyacinth macaw CHD-NW (Griffiths & Tiwari 1995; see figure 2).

A semi-nested PCR on captive Spix's macaw DNA with P1, P2 and P3 primers produces a CHD-NW derived fragment in both sexes. This provides a positive control as its presence indicates that the PCR has not failed because of technical problems or inhibitors in the DNA samples. The same PCR will also amplify the W-linked CHD-W fragment only in female birds. The problem remains that both PCR products are of identical size. The Spix's macaw W-linked product does contain a unique *DdeI* site. Cutting the female PCR product produces three fragments: uncut CHD-NW (104 bp) and CHD-W cut into two fragments (73 and 31 bp). In males only CHD-NW is amplified and this remains at 104 bp after cutting with *DdeI*. This makes males and females easily separable in the Spix's macaw (Griffiths & Tiwari 1995).

If the same test is to work on other bird species then two criteria must be met. The first is whether the PCR primers will amplify both CHD genes in other bird species. The Spix's macaw test used the tiny amounts of DNA extracted from feathers so a semi-nested PCR was required. This used three primers which are aligned to the mouse and chicken CHD nucleotide sequences in figure 2. The primer sites are highly conserved, there is no difference between the chicken genes and a solitary difference between the mouse and chicken in the 5′ region of the P2 site. Theoretically, the primers should anneal to other bird species and, if a reasonable amount of DNA is available (greater than 50 ng), a single pair of primers should provide sufficient amplification.

A second requirement for the test is that the PCR products can be separated using a restriction endonuclease. In the Spix's macaw the *DdeI* enzyme cuts CHD-W, but not CHD-NW. Figure 2 shows that this discrimination would also occur in the chicken. However, the *DdeI* cutting site CTNAG is not present in the CHD-NW of Spix's macaw (CTNGG) nor the chicken (CANAG) for different reasons. This suggests that the *DdeI* site is open to mutation so this form of discrimination is unlikely to be conserved. Other discriminatory sites are available: *DdeI* and *MaeII* sites are unique to CHD-W and the *HaeIII*, *MboII* and *XhoI* sites to CHD-NW and can be considered the first option. If these fail the CHD-W and CHD-NW PCR fragments can be cloned and sequenced so discriminatory sites can be discovered.

The theory we have presented suggests that a sexing test based on both avian CHD genes should work on many other bird species. Does this work in practice? The birds selected for trial are from across the avian class: chicken (five individuals), Marbled murrelet (18), kestrel (eight), Marsh harrier (28), bee-eater (four), one pair of six species of Strigidae owls from different genera (see §2), starling (five) and African marsh warbler (5).

The primers amplify a PCR product of 110 bp in all of the birds using primers P2 and P3 on 50–100 ng of genomic DNA extracted from blood. Figure 3 illustrates this for three bird species (lanes 1 and 2) but

```
MOUSE     CHD1      AGA TAT TCT GGA TCT GAT AGT GAT TCA ATC TCG GAA
CHICKEN   CHD-NW    --- --- --- --- --- --- --- --C --C --- A-A ---
SPIX      CHD-NW                                    C --C --- --- ---
CHICKEN   CHD-W     --- --- --- --- --- --- --- --- --C --- --A ---
SPIX      CHD-W                                     C --C --- --A ---
P1                    A TAT TCT GGA TCT GAT AGT GAY TC     DdeI
P3                  AGA TAT TCC GGA TCT GAT AGT GA

MOUSE     CHD1      AGG AAA CGG CCG AAG AAA CGT GGG CGA CCC CGC ACT
CHICKEN   CHD-NW    --A --- --- --A --A --G --- --A A-- --T --A --C
SPIX      CHD-NW    --- --- --- --A --A --G --- --A A-- --A --A ---
CHICKEN   CHD-W     --A --- --A --A --A --- --- --A --- --A --A ---
SPIX      CHD-W     --A --- --A --A -GA --- --- --A --- --A --A ---
                              HaeIII  M

MOUSE     CHD1      ATC CCT CGG GAG AAT ATT AAA GGA TTT AGT GAT GCG GAG
CHICKEN   CHD-NW    --T --- --A --A --- --- --- --- --- --- --A ---
SPIX      CHD-NW    --T --- --A --A --- --A
CHICKEN   CHD-W     --T --C --T --A --C --- --- --- --- --- --- --A ---
SPIX      CHD-W     --T --- --T --A --- ---
P2                                        TTT CCT AAA TCG CTA CGT CT
```

Figure 2. The nucleotide sequence of part of a single CHD1 gene isolated from the mouse and the two homologous genes from the chicken and spix's macaw all arranged as putative codons. Dashes denote nucleotides shared with the mouse CHD1 sequence. The primers designed are shown on the diagram as P1, P2 and P3. A letter **M** under the second line of aligned sequences indicates a non-synonymous mutation in the spix CHD-W. The *DdeI* (CTNAG) and *HaeIII* (GGCC) sites are underlined.

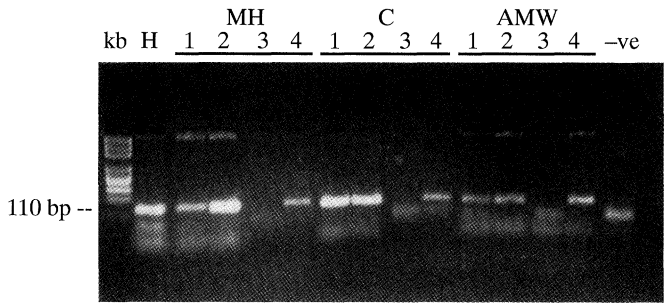

Figure 3. Sex identification in the marsh harrier (MH), chicken (C) and African marsh warbler (AMW) done using an identical reaction. For each species genomic DNA of male and female birds was subject to PCR with primers P2 and P3. PCR products of 110 bp are visible in lanes 1 (male) and 2 (female). In lanes 3 and 4 these male and female PCR products have been cut with *HaeIII* before electrophoresis. In lane 3 the male PCR product has cut with *HaeIII* so the 110 bp band has disappeared. In the female, in lane 4, *HaeIII* cannot cut the CHD-W product so the band remains at 110 bp. The 'kb' lane contains a '1 kb DNA ladder' (BRL), the 'H' lane is PCR reaction with P2 and P3 done on human genomic DNA. The negative lane contains an uncut negative PCR reaction whose artefactual product is easily distinguishable from the uncut genomic PCR product.

also includes amplification from human DNA. This shows that tests using P2 and P3 are open to human DNA contamination so appropriate precautions must be taken.

The *HaeIII* restriction enzyme cut the CHD-NW fragment alone in all 13 species. Figure 3 shows that the 110 bp CHD-NW male PCR product has gone as it is cut into two fragments (45 bp, 65 bp) which are not easily visible on the gel (lane 3). In females CHD-W is uncut by *HaeIII* so remains at 110 bp (lane 4). The discrimination using *HaeIII* provided correct sex identification in all individuals.

4. DISCUSSION

Here we show that the Great tit GT1 sequence is an intron of a CHD gene, the first avian W-linked gene to be described. The name CHD is derived from a closely related gene discovered by Delmas *et al.* (1993) in the mouse. The CHD gene contains three functional domains: a chromatin organization-modifier, a helicase–ATPase and a DNA binding region. The first two motifs have never previously been found within a single protein, they occupy separate genes such as the *Polycomb* (chromo) and *Brahma* (helicase–ATPase) developmental genes. These two examples have antagonistic effects that help control the development of *Drosophila melanogaster* (Singh 1994). How the two motifs interact within a single gene will be interesting to discover. The third motif, the DNA binding site, appears to target (A+T)-rich motifs, through DNA recognition and binding via the minor groove of DNA (Stokes & Perry 1995).

A comparison between the bird genes either by nucleotide sequence analysis or by Southern blotting shows that the two avian CHD genes are both similar and highly conserved. Benton (1990) estimates that the split between the chicken and the remaining birds we considered occurred around 79 million years ago. According to the phylogeny of Sibley *et al.* (1988)

this represents the earliest split in the avian class suggesting that the CHD-W gene already occupied the W-chromosome in the common ancestor of Eoaves and Neoaves.

Delmas *et al.* (1993) have previously demonstrated a similar nucleotide sequence conservation of the CHD gene within the mammals. A comparison of short amino acid sequences between three bird species and the mouse showed the identity varied between 85% (41AA) and 97–100% (37AA) in two different regions. This confirms the conservation of CHD over the 300 million years (Benton 1990) since the separation of the two classes. A possible difference between CHD in birds and mammals is the number of genes that occur in each individual. In birds there appears to be two copies while there appears to be a single CHD gene in the mouse (Delmas *et al.* 1993; Stokes & Perry 1995).

The reason for this difference may be caused by the positions of the genes. Southern blotting has shown that CHD-W lies upon the W chromosome but CHD-NW could occupy a place on either the Z chromosome or an autosome. The sequence similarity of the two avian genes and the dosage importance of a gene like CHD (Singh 1994) suggest that CHD-NW may occupy the Z chromosome. A paper by Baverstock *et al.* (1982) suggests there is no specific dosage control associated with a gene being present on the Z chromosome. This means a male (ZZ) bird would produce twice the amount of Z-linked CHD-NW protein than a female (ZW). However, if Z and W both contained functionally similar CHD genes then the dosage of the CHD protein would be identical in both sexes. This level of functional conservation may also account for the lack of nucleotide sequence divergence between CHD-W and CHD-NW.

The first W-chromosome linked DNA was isolated by Tone *et al.* (1982) from the chicken. Since then, a number of other W-linked avian sequences have been discovered (see, for example, Griffiths & Holland 1990; Rabenold *et al.* 1991; Griffiths & Tiwari 1993). In all but one case, described later, these DNA fragments appear to be non-functional repeats. For instance, the related *XhoI* and *EcoRI* fragments in chicken may comprise 70–90% of the W chromosome (Saitoh *et al.* 1991). This repeat and others in the Lesser black-backed gull can be used to sex birds by the rapid dot blotting technique (Griffiths & Holland 1990). Other less repetitive W-chromosome markers can be used to sex birds either by probing Southern blots (Rabenold *et al.* 1991) or through the use of PCR (Griffiths & Tiwari 1993).

The principal problem with all non-functional W-linked DNA is the speed with which they evolve. The chicken *XhoI* repeat is fairly typical. Through low stringency hybridization to a Southern blot it can be used to sex the turkey (*Meleagris gallopavo*) and the pheasant (*Phasianus versicolor*; Saitoh *et al.* 1991). These bird species are closely related to the chicken by being members of the family Phasianidae. By contrast, the functional CHD-W region described here is 96% (3/67 figure 2) identical between chicken and Spix's macaw and this only drops to 86% between the chicken CHD-W and the mouse CHD1 (15/110 figure 2). This level of conservation means that the chicken CHD-W probe can be used on Southern blots to sex birds from all over the class Aves.

The only exception to the non-functional avian W-linked sequences is DZWM1 which is a putative gene, cloned from a cDNA turkey library. Like CHD-W this gene appears to be sex linked in many bird species. Unfortunately, so little information has been published in the papers that describe DZWM1 that the nature of the gene remains unknown (Halverson 1990; Dvorák *et al.* 1992; Halverson & Dvorak 1993).

For sexing large numbers of birds Southern blot analysis is slow and expensive. The technique that we have used is based on a PCR using P2 and P3 primers followed by a *HaeIII* digestion of the of the amplified product. The digestion distinguishes between the CHD-W product which is uncut and the CHD-NW which is cut. The technique will work to sex 14 bird species that span the class Aves. The primers target a highly conserved region so are likely to be 'universal' to the birds, but the discriminatory *HaeIII* site which cuts CHD-NW but not CHD-W shows no real reason to be conserved. If *HaeIII* does fail to be discriminatory other cutting sites have been suggested or the CHD-W and CHD-NW PCR products can easily be sequenced to look for an alternative.

The CHD based test appears to be fairly solid but the chances of a peculiar mutation in some bird species is not impossible. Cases concerning SRY/Sox3 genes on the sex chromosomes in mammals supports this claim. In two species of the vole *Ellobius* males have neither a Y chromosome nor an Sry gene (Just *et al.* 1995). In a second case, four species of *Akodon*, the mole vole, have 15–40% of fertile females with XY chromosomes and an Sry gene (Bianchi *et al.* 1993). These examples are particularly strange in that the SRY gene is accepted as the gene that determines sex throughout the mammals. In neither case would the detection of Sry reliably inform you of the animals sex.

These examples from the Muridae may never occur with the CHD genes of birds. However, it does suggest that sex identification by the amplification of CHD-W and CHD-NW should always be validated by a test on several individuals in a new species before it is applied.

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